# COMPLEMENTARY DNA CLONING AND PARTIAL MOLECULAR CHARACTERIZATION OF CITRUS TRISTEZA AND CITRUS VARIEGATION VIRUSES

By

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TABLE OF CONTENTS

Abstract of Dissertation Presented to the Graduate School
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# COMPLEMENTARY DNA CLONING AND PARTIAL MOLECULAR CHARACTERIZATION OF CITRUS TRISTEZA AND CITRUS VARIEGATION VIRUSES

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The RNA of citrus tristeza virus (CTV), a closterovirus with a 20 kilobase (kb) single-stranded RNA genome, and the RNA of citrus variegation virus (CVV), an ilarvirus with a single-stranded tripartite RNA genome, were used to prepare complementary DNA libraries. Selected clones were confirmed to be viral specific by hybridization analyses.

The CVV-cDNA library consisted of 76 cDNA clones. One clone, approximately 1750 bases in length, was complementary (Northern blot analysis) to RNA 3 and RNA 4 of the CVV genome. Another clone of over 800 bases hybridized with the CVV RNA 2 and the CVV RNA 4a. This indicated that CVV 4a may be a subgenomic product of RNA 2. These clones were used as probes to characterize the homology between two strains of CVV and one strain of citrus leaf rugose virus.

Libraries of CTV-cDNA clones were prepared to a mild strain T-30, and a severe strain T-36. The T-30 library consisted of over 200 clones. A clone designated 30F6, which shared homologous regions with most of the clones in the library, was partially sequenced. The library of clones prepared from T-36 RNA consisted of over 500 clones. Two clones, which shared homology for approximately 2200 bases, represented nearly 70% of the CTV genome. The 5' and 3' orientation of the clones as compared with the CTV-RNA was determined. Portions of the clone P6A6, which were proximal to the 3' end of the CTV-RNA, were sequenced. The primary structure of a protein encoded by an open reading frame of at least 327 bases was predicted. Within another subclone of P6A6, an area of poly (A) bases was found and this region may be similar to an intergenic region of brome mosaic virus RNA 3. A 5000 base CTV-cDNA fragment was subcloned into open reading frame expression vectors and transformed into *Escherichia coli*. The resulting blue colonies were selected and confirmed to contain CTV-cDNA inserts by restriction and hybridization analyses. Four distinct trihybrid fusion proteins were identified and one was purified for use as an immunogen.

# CHAPTER 1
# INTRODUCTION

Citrus tristeza virus (CTV) is a member of the closterovirus group (4). There are three distinct classes of closteroviruses which are grouped according to particle lengths of 720-800 nm, 1250-1450 nm, and 1650-2000 nm. The virions are approximately 11 nm wide. The closteroviruses are aphid transmitted in a semipersistent manner, although white flies are the reported vector for lettuce infectious yellows virus, a member of a possible new subgroup of closteroviruses (18). The closteroviruses, as a group, have not been well characterized. Some of the inherent problems which retard research progress with these viruses include their narrow host ranges, the absence of local lesion assay plants, and the fact that virions are phloem-limited, generally in low titers, and easily sheared. Inclusion bodies, which contain viral particles, are found in the phloem of infected hosts (4). No proteins in the inclusion bodies, except for the viral coat protein, have been identified as being viral-encoded proteins.

Citrus tristeza virus is distributed worldwide and is economically the most important viral disease of citrus. There is a wide range of symptoms caused by CTV strains (53). A mild strain may cause only vein clearing, while a more severe strain can cause stem pitting on sweet orange (*Citrus sinensis* (L.) Osbeck) or grapefruit (*Citrus paradisi* Macf.). The severity of stem pitting can range from minor importance to very severe and debilitating to the diseased

tree. There are CTV strains in Brazil and other countries, but not in Florida, that cause severe stem pitting on either sweet orange or grapefruit. If these severe stem pitting strains are introduced into Florida, there is the potential for widespread losses not only to 20 million trees already established on sour orange, but also to trees established on CTV-tolerant rootstocks. The seedling yellows of sour orange (*Citrus aurantium* (L.)), grapefruit, and some lemon varieties (*Citrus limon* (L.) Burm. f.) or the stunting of trees are other symptoms which can be associated with severe strains of CTV. Citrus established on sour orange rootstock is susceptible to strains of CTV that cause quick decline, leading to the death of the infected trees.

The maintenance of sour orange as a rootstock is important in Florida because trees on this rootstock are relatively tolerant to citrus blight and cold damage. The planting of citrus trees on sour orange rootstock has been increasing in Florida throughout the last decade (30), and there are currently epidemics of CTV strains which cause quick decline. This has resulted in the death of many trees on sour orange rootstock (6). Severe strains of CTV endemic to Florida are constraints on the widespread use of sour orange rootstock.

The control of CTV is difficult. Most work has centered on the production and use of tolerant or resistant citrus scions or rootstocks. Some work with virus diseases has shown that cross protection might be a useful control strategy. Cross protection is the phenomenon whereby a mild strain of the virus protects the plants from severe strains. Cross protection of citrus with mild strains of CTV has been used on a commercial basis in Brazil (12); however, more progress is needed on determining the traits which make a

virus strain potentially useful in a cross protection strategy (30). The selection of mild CTV strains for cross protecting citrus from severe strains of CTV has been done empirically only through greenhouse and field trials. The molecular basis for the phenomenon of cross protection is poorly understood. The identification of the proteins encoded by the virus will be a prerequisite to determine if viral-encoded proteins are involved in cross protection. The identification of viral products involved in cross protection could be an important step towards establishment of systematic strategies of crop protection.

The transcription and translation strategy of CTV as well as the 5' and 3' termini structures are unknown. While *in vitro* translation of CTV has shown that other proteins are translated from CTV-RNA (37), no antisera have been made to the viral-encoded non-structural proteins. The only well characterized CTV-encoded protein is the capsid protein (31,32). There is a need to identify the viral-encoded proteins and to assign functions to these proteins. The sequencing of a viral genome is one means to identify open reading frames and to determine the primary structure of viral-encoded proteins (27). Homology searches have been used successfully to assign putative functions to proteins even though the proteins are encoded by viruses that are in different groups (8).

Citrus variegation virus (CVV) is endemic to Florida. It is a member of the ilarvirus group which contains viruses that have been well characterized. The 3' termini of the several ilarviruses have been shown to be folded into tRNA like structures (56). Since CVV is a citrus virus that is relatively easy to purify and is known to lack poly (A) sequences on the 3' termini, it was chosen as a model virus for preliminary study. Complementary DNA cloning and

other techniques useful for molecular characterization of plant viruses were first attempted with CVV and then applied to CTV.

The objectives of this research include the production of CTV-cDNA clones to provide a means to study the genomic organization of the virus, the identification of viral-encoded proteins, and the development of rapid biochemical assays to identify variation between strains of CTV. The preparation of a library of CTV-cDNA clones to study the genomic organization of CTV is important because CTV RNA is difficult to purify in sufficient quantities for such studies. The CTV-cDNA clones can be inserted into RNA transcription and protein expression vectors to synthesize viral products including the RNA and the non-structural proteins. The expression of plant viral proteins in *E. coli* containing plant viral cDNA sequences has been demonstrated for proteins of tobacco mosaic virus, papaya ringspot virus, and other plant viruses (45,38,33). Fusion proteins consisting of amino acid sequences encoded by foreign DNA and the amino acid sequences of the ß-galactosidase protein were used to produce antisera to the foreign protein (54,55). The sequencing of cDNA plant viruses has been used to predict the primary structure of the viral-specified proteins and to determine the structural organization of the viral genomes (9,10,11,27). These molecular techniques were applied to characterize partially the viral genome and proteins of CTV. The long range goals of this project include the characterization of the proteins encoded by the CTV genome and the determination of the transcription and translational strategy of the virus.

CHAPTER 2
THE RNA SPECIES OF CITRUS VARIEGATION VIRUS CHARACTERIZED WITH COMPLEMENTARY DNA CLONES.

## Introduction

Infectious variegation previously was considered part of the psorosis disease complex (52), but after citrus variegation virus (CVV) was purified and characterized, it was determined to be an ilarvirus (21). Citrus leaf rugose virus (CLRV) and CVV are serologically related ilarviruses which cause diseases that are distinct from those caused by the psorosis viruses (20,22).

The genome of CVV consists of three single-stranded messenger sense RNAs, which are designated RNA 1, 2, and 3 in order of decreasing size and are encapsidated separately in icosahedral particles. Two additional RNA species, designated RNA 4 and 4a, are present in viral RNA preparations (24). The RNA 4a has not been reported in CLRV or the other ilarviruses. The CVV RNA 1, 2, and 3 require the RNA 4 + 4a fraction or coat protein to be infectious (24). The coat proteins of other ilarviruses or alfalfa mosaic virus (AlMV) can be interchanged with the coat protein of CVV to cause a mixture of the RNA 1, 2, and 3 species of these viruses to be infectious (23,25). The coat proteins of AlMV and tobacco streak virus (TSV) are interchangeable despite the lack of homology between the coat proteins (11). The coat protein binds preferentially to sequences in the 3' terminal region of the RNA species, and this binding has been hypothesized to be required for viral replicase recognition of viral RNAs (26).

The ilarviruses and the AlMV group have been proposed as one genus of the family of tripartite viruses, the Tricornaviridae (49). Several members of

the ilarvirus and AlMV groups are well characterized. The complete tripartite genome of the AlMV (strain 425) is sequenced. The RNA 1 and 2 each contain a single open reading frame (9,10). The RNA 3 of AlMV and TSV contain two open reading frames (5,11), but only the 5' proximate coding region is translated. The RNA 4 is a subgenomic messenger RNA for the coat protein and is homologous to the 3' proximal end of RNA 3. The proteins translated from RNAs 1-4 are designated P1-4, respectively.

This is the first report on cDNA cloning of CVV RNA. Two cDNA probes were used to compare homology of the RNA species. These probes were also used to test their efficacy in detecting a second isolate of CVV and also CLRV.

## Materials and Methods

### Virus purification

The viral isolates, CVV-1 and CLRV previously described by Garnsey (20,21), and the isolate CVV-FM, which causes severe symptoms in citron, grapefruit, and *C. excelsa*, were maintained in greenhouse cultivated citron plants. The virions were purified by a method described by Garnsey (21) except that two cycles of calcium phosphate gel treatment were used for clarification of the crude extract.

### Materials used for cDNA cloning and analysis of the clones.

Restriction enzymes, DNA polymerase I, polyadenine polymerase, terminal deoxynucleotidyl transferase, T-4 ligase, phenol, RNA molecular weight standards, lambda RF-DNA, and phiX 174 Hae III fragments were all from Bethesda Research Laboratories, Inc. (Gaithersburg, MD 20877). Deoxynucleotides, ribonucleotides, and 5-bromo-4-chloro-3-indolyl-ß-D-galactosidase (X-gal) were obtained from Boehringer Mannheim (Indianapolis, IN 46250). The *E. coli* strain JM 83 and the plasmid pUC-19 were described by Vieria and Messing (50). The AMV reverse transcriptase was from Seikaguki

(St. Petersburg, FL 33702). The radioisotope $[32]P$ deoxycytosine triphosphate (dCTP) was from Amersham (Arlington Heights, IL 60005). Nitrocellulose membranes and NA-45 cellulose membranes were from Schleicher and Schuell (Keene, NH 03431).

Isolation and polyadenylation of the viral RNA

The RNA was extracted from purified virus by treatment with 0.1% sodium dodecyl sulfate (SDS) in 50 mM tris(hydroxymethyl)aminomethane (Tris), pH 8.0, for 5 minutes at 60 C, extracted with phenol, phenol:chloroform, and chloroform (43). One-half volume of 7.5 M ammonium acetate and three volumes of absolute ethanol were added to the preparation before RNA precipitation by centrifugation (34). The CVV-RNA pellet was rinsed with 70% ethanol and dried. The RNA was resuspended in sterile water and the quantity was estimated by spectro-photometric analysis at A=260 nm with a path length of 1 cm using extinction coefficient of 25.

The RNA was polyadenylated using poly (A) polymerase to catalyze a 3' tailing reaction following the protocol of Devos et al. (14). The reaction mixture was extracted with phenol:chloroform and the RNA was recovered by ethanol precipitation.

Synthesis and Cloning of cDNA

The hybrid cDNA:RNA cloning procedure described by Cann *et al.* (7) was used as the basis for the construction of cDNA library of CVV RNA. The vector, pUC-19, was cut with the Pst I restriction enzyme and tailed at the 3' termini with deoxyguanidine (dGTP) using terminal deoxynucleotidyl transferase to catalyze the reaction. The reaction was terminated by the addition of ethylene diamine tetraacetic acid (EDTA), and the reaction mixture was treated with phenol:chloroform and the nucleic acids were ethanol precipitated.

The cDNA synthesis was primed by the addition of oligodeoxythymidylic acids (oligo $(dT)_{12-18}$) to 2-4 ug of polyadenylated CVV RNA. The reaction was terminated by the addition of EDTA and the reaction mixture was extracted with phenol:chloroform. The unincorporated nucleotides were removed by spin column chromatography (34) using Bio-gel P60 from Bio-Rad (Richmond, CA 94804) as the column matrix gel, and the fractions containing incorporated isotope were ethanol precipitated. The cDNA:RNA hybrids were then tailed with dCTP using terminal deoxynucleotidyl transferase to catalyze the reaction. The procedures used to stop the reaction and remove the unincorporated nucleotides were repeated. The cDNA:RNA hybrid preparations were resuspended with water and annealed to the poly (G) tailed pUC-19 using the conditions described by Cann *et al.* (7). After the cDNA:RNA hybrids were annealed to the pUC-19 vector, they were used to transform cells of *E. coli* strain JM 83 which had been made competent using a calcium chloride treatment (34). The bacterial cells were grown on Luria-B medium containing ampicillin at 50 ug/ml and 3-indolyl-ß-D-galactosidase at 40 ug/ml. The resultant white and light blue colonies were assayed for cDNA inserts of CVV RNA by hybridization with $[32]$P dCTP labelled first-strand cDNA transcribed from randomly primed CVV RNA (34).

Analysis of the cDNA clones.

The plasmids of the CVV-cDNA clones were purified using an alkali purification procedure (41), cut with various restriction enzymes, run on agarose gels, and the DNA transferred to nitrocellulose membranes using protocols described by Maniatis *et al.* (34). Selected cDNA inserts were recovered from the agarose gel with NA-45 cellulose membranes (17). These cDNA inserts were $[32]$P labeled by nick translation (34), and used as probes for the detection of total CVV RNA or for CVV RNA which had been glyoxalated

and separated into its RNA species by agarose gel electrophoresis and transferred to nitrocellulose filters (Northern blot analysis). The hybridization solution contained sodium chloride and sodium citrate (6X SSC), 5X Denhardt's solution, and salmon sperm (34), and the filters were hybridized overnight at 50 C or 68 C.. The filters were rinsed for 5 minutes at room temperature and then for 15 minutes at either 50 C or 68 C in 2X SSC containing 0.5% SDS. The final rinse was for 15 minutes at either 50 C or 68 C in 0.5X SSC containing 0.5% SDS.

## Results

The species of CVV RNA were estimated to be 3520, 2990, 2350, 1120, and 890 bases in size for the RNA 1, 2, 3, 4, and 4a, respectively (Fig. 1). These are equivalent to Mr 1,196,000, 1,015,000, 800,000, 380,000, and 304,000, respectively. The molecular weights of the CLRV RNA species were nearly identical to their CVV-RNA counterparts. No RNA 4a species was detected in the CLRV preparations.

After transformation of *E. coli* with the cDNA:RNA hybrids annealed with the pUC-19 plasmids, 76 of the 105 white and light blue colonies were positive in colony hybridization tests. The average length of the cDNA inserts for 27 clones was approximately 500 bases. Four of the cDNA inserts were approximately 1750 bases in length, and one of these clones (CVV-34) hybridized with RNA-3 and RNA-4 of CVV in Northern blot analysis at 68 C (Fig. 2). These four clones were similar in size and had common restriction sites. Restriction digests for the clone CVV-34 analyzed on agarose gels are shown in Figure 3, and the position of the known restriction sites are shown in Figure 4. The two fragments of 1100 and 650 bases that resulted when the plasmid was cut with Hind III and Bam HI restriction endonucleases were purified using agarose gel electrophoresis and NA-45 cellulose membranes. Both the

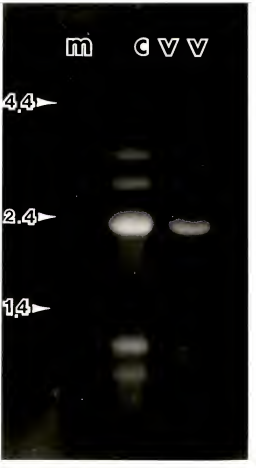


Figure 1. Separation of RNAs 1, 2, 3, 4 and 4a after glyoxalation and electrophoresis on agarose gels. Ethidium bromide was used to stain the RNAs. The lane m contains single-stranded RNA standards of 4.4, 2.4, and 1.4 kilobases which are indicated with arrows from top to bottom, respectively. RNAs extracted from the CVV-1 isolate are in concentrations of 3 ug and 1 ug per lane (quantified by optical density) in the middle and left lanes, respectively.

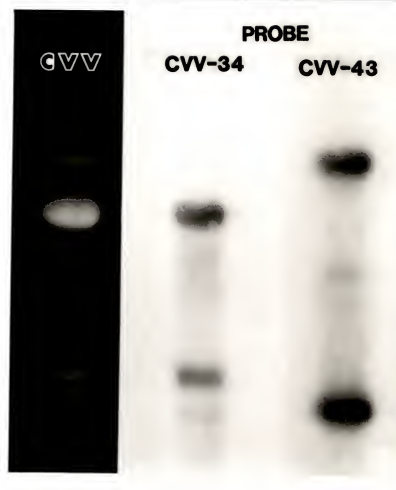


Figure 2. Separation of CVV-1 RNAs after glyoxalation and electrophoresis on an agarose gel. The lane CVV contains the CVV-1 RNAs stained with ethidium bromide. Lanes CVV-34 and CVV-43 are autoradiograms of CVV-1 RNAs which were transferred to nylon membranes and hybridized at 68 C with $[32]$P-labelled clones CVV-34 and CVV-43. Probe CVV-34 hybridized with RNAs 3 and 4, and probe CVV-43 hybridized with RNAs 2 and 4a.

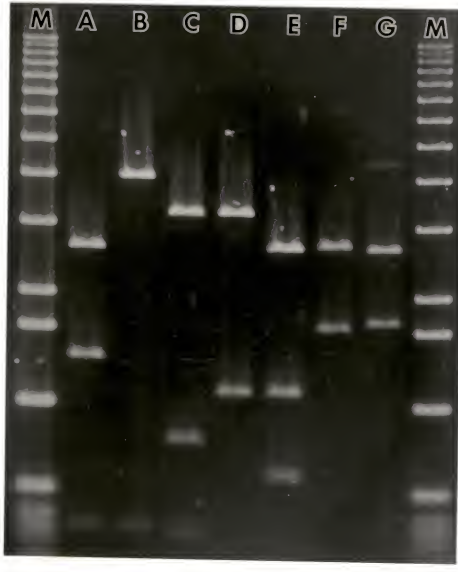


Figure 3. Agarose gel separated restriction digests of the cDNA clone CVV-34 stained with ethidium bromide. Lanes M are markers, lane A is a digest with the restriction enzymes Hind III and Eco RI, lane B Eco RI, lane C Eco RI and Bam HI, lane D Bam HI, lane E Hind III and Bam HI, lane F Pst I, and lane G Hind III and Sst I. The 2700 base fragment in lanes A, E, F, and G is the pUC-19 vector fragment. The cDNA clone is in the vector pUC-19 and was inserted at the Pst I site. The Hind III restriction site in the polylinker region is adjacent to the 3' terminus.

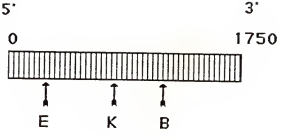


Figure 4. Endonuclease restriction map of the CVV-1 RNA 3 cDNA clone CVV-34. The Eco RI site is at base 320, the Kpn I site is at base 800, and the Bam HI site is at base 1100. The cDNA clone is in the vector pUC-19 and was inserted at the Pst I site. The Hind III restriction site in the polylinker region is adjacent to the 3' terminus.


Figure 5. Hybridization at 50 C of $^{[32]}$P-labelled clones of CVV-34 (left) and CVV-43 (right) with Northern blots of RNAs from isolates CVV-FM, CVV-1, and CLRV, lanes A, B, and C, respectively. The autoradiogram was overexposed in an attempt to detect hybridization with CVV-FM isolate and CLRV.

fragments were nick translated using [32]P dCTP as a label and they hybridized to both the RNA 3 and RNA 4 in Northern blot analysis at 68 C (data not shown but identical with the hybridization pattern of CVV-34 probe in Fig. 2). Using these clones to screen a second library of CVV clones, the 650 base fragment hybridized with 18 clones and the 1100 base fragment hybridized with 7 of the clones.

Another CVV clone, CVV-43, which had an insert about 800 bases in length, hybridized with the RNA 2 and the RNA 4a of CVV in Northern blot analysis at 68 C (Fig. 2). No internal restriction site has been determined for this clone.

The nick translated clones CVV-34 and CVV-43 were used as probes to attempt the detection of the RNA of the CVV-FM isolate and CLRV using conditions that were less stringent than those used in detection of the RNA of the homologous strain CVV-1. The hybridization of these probes and the washing of the filters were done at 50 C, and even when the filters were overexposed, no hybridization with CVV-FM or CLRV could be detected (Fig. 5).

## Discussion

The DNA:RNA hybrid method of cDNA cloning as described by Cann *et al.* (7) has been used successfully to clone viruses which contain poly (A) sequences on their 3' termini (7,47). Because CVV RNAs lack 3' poly (A) sequences, the CVV RNA was polyadenylated *in vitro* which permitted oligo dT to be used as the primer for the first strand cDNA synthesis. The poly (A) polymerase reaction requires the presence of a free 3' hydroxyl group. Since the 3' termini of ilarviruses are known to fold into a secondary structure resembling tRNAs, it was not known if CVV could be polyadenylated. The success of the CVV cloning demonstrated that cloning methods that rely on the

presence of a poly (A) 3' termini can be adapted to single-stranded RNA viruses that normally lack a poly (A) tail.

The orientation of the CVV-34 clone was resolved on the basis of the hybridization of the 650 and 1100 base fragments with RNA 4. Since the RNA 4 size was estimated to be 1120 bases, the 650 base fragment was determined to be the 3' proximal region. The screening of the second CVV library showed that three times as many clones hybridized with the 650 base fragment as with the 1100 base fragment. The 3' end of the RNA species would be expected to be in greater abundance because of the cDNA cloning method used.

The size estimates for the RNA species are approximately 10% higher than the previous estimates (24). The estimates in this report were made by comparing CVV RNA species and ss-RNA standards which had been denatured with glyoxal. These estimates are similar to the sizes that are reported for other closely related viruses (5,9,10,11). After CVV RNA was separated on an agarose gel and transferred to a nitrocellulose filters, the CVV-cDNA clones were identified by hybridization analysis as complementary with specific CVV RNA species. Using this method, the RNA 4a species was homologous to the RNA-2. Whereas the 3' end of the RNA species of AlMV are highly homologous (56), it is not likely that RNA 4a hybridized because of such limited shared homology. When using lower stringency conditions (Fig. 4) with the CVV-43 probe, only RNA 2 and RNA 4a were detected. It is possible that the RNA 4a is a subgenomic species, since it has the potential to encode a protein of more than Mr 30,000.

The use of cDNA probes to detect viruses appears to be very specific. The viruses CVV-1, CVV-FM, and CLRV are serologically related. Nevertheless, no homology between the viruses could be detected with the limited number of

clones tested even when using less stringent conditions for hybridization and washing. This confirms that cDNA probes can be highly specific for virus and strain identification.

CHAPTER 3
THE COMPLEMENTARY DNA CLONING OF CITRUS TRISTEZA VIRUS AND THE PARTIAL SEQUENCING OF SELECTED CLONES

## Introduction

Citrus tristeza virions consist of about 20 kilobases of single-stranded messenger-sense RNA packaged in long flexuous rods approximately 2000 nm X 11 nm in size (4). Citrus tristeza virus (CTV) is aphid-transmitted in a semipersistent manner and is phloem-limited. Inclusion bodies, which contain virions, are also present in the phloem of citrus infected with CTV. There is little information available concerning the genomic organization or the proteins encoded by CTV. The only well characterized CTV encoded-product is the coat protein, which is approximately Mr 23,000 and thus represents approximately 3% of the estimated coding capacity of the viral genome (31). A protein of the Mr 21,000, which is also found in purified viral preparations, cross reacts with CTV antisera, and peptide mapping indicates it is closely related to the capsid protein. Polyclonal antisera produced against several strains of CTV react with all strains of CTV tested. Polypeptide mapping indicates that the amino acid sequence of the coat protein is highly conserved among the Florida strains of CTV (32), even though the strains have different biological activities. Proteins of various sizes are translated by the CTV RNA *in vitro*, and the coat protein can be immunoprecipitated from the translation products (37). The coat protein is the only CTV encoded-product for which a function is known.

Because CTV is phloem-limited and present in infected tissue in relatively low titers, the cDNA cloning of the viral RNA is an important amplification

step for studying the CTV genome. The primary sequence of the viral genome can predict the size and primary structure of viral-encoded proteins. The purpose of this study was to prepare a library of cDNA clones from CTV RNA. Portions of the cDNA clones were sequenced to determine the primary structure of viral-encoded proteins and the probable translation strategy of the virus.

## Materials and Methods

### Materials

The restriction enzymes, DNA polymerase I, polyadenine polymerase, cloned MLRV reverse transcriptase, terminal deoxynucleotidyl transferase, T-4 ligase, Klenow fragment DNA polymerase I, the 1 kilobase ladders and the 123 base ladders used were from Bethesda Research Laboratories, Inc. (Gaithersburg, MD 20877). Dideoxy-nucleotides, RNasin, pGEM™ vectors, T7 and SP6 primers and polymerases were obtained from Promega Biotec (Madison, WI 53711). *Escherichia coli* strain JM 83 and the vector, pUC-19 were those described by Vieria and Messing (50), and nitrocellulose and NA-45 cellulose membranes were from Schleicher and Schuell (Keene, NH 03431).

### Virus purification

A mild strain of CTV, designated T-30, and a severe seedling yellows strain of CTV, designated T-36, were used in this study. These strains, which were previously described by Rosner *et al.* (44), were maintained in either citron or *Citrus excelsa* (L.) Wester that were grown in greenhouses at approximately 30 C. The virions were purified by the method described by Lee and Calvert (31) with the following modifications to maximize yield and minimize damage to the viral RNA. The modified extraction buffer contained 2% driselase, 8% sucrose, 5 mM 2-Mercaptoethanol, 0.4% TritonX-100 in 0.1 M sodium citrate buffer, pH 7.0. The steps involved in freezing of the preparation and chromatography

with the Bio-gel A-15 column were eliminated. The first cesium sulfate gradient was slightly modified in that each tube contained 22.5 ml of the supernatant layered onto 15 ml of 2 molal cesium sulfate in 50 mM tris(hydroxymethyl) aminomethane (Tris). The gradient was formed by 12 hours of centrifugation in a Ti 60 rotor at 45,000 rpm. After the second cesium sulfate gradient, the virion containing fraction was dialyzed 3 times against 50 mM Tris containing 8% sucrose. The CTV RNA was purified by treatment of the viral preparation with 0.1% sodium dodecyl sulfate (SDS) in 50 mM Tris, pH 8.0, for 5 minutes at 60 C, then the preparation was chilled on ice and extracted twice with phenol:chloroform (43). One-half volume of 7.5 M ammonium acetate and two volumes of absolute ethanol were added to preparation, and after storage for several hours at -20 C, the RNA was collected by centrifugation. The precipitated CTV RNA was resuspended in sterile water and the quantity was estimated by spectrophotometric analysis at A=260 nm with a path length of 1 cm using extinction coefficient of 25.

The RNA was polyadenylated (14) using poly (A) polymerase to catalyze a 3' tailing reaction. The reaction mixture was treated with phenol:chloroform and the RNA was recovered by ethanol precipitation.

Synthesis and cloning of cDNA

A hybrid cDNA:RNA cloning procedure was used to prepare cDNA libraries of CTV RNA (7). Several modifications to the procedure were made (see Appendix A), especially the elimination of many of the phenol extraction steps. The plasmid pUC-19 purified by the procedure of Garger *et al.* (19), was used as the cloning vector. The vector was cut at the Pst I restriction site and poly (G) tails were added to the 3' termini using terminal deoxynucleotidyl transferase to catalyze the reaction.

The cDNA synthesis was initiated using oligo $dT_{12-18}$ primer. The reaction conditions were 50 mM Tris, pH 8.3, 75 mM KCl, 8 mM $MgCl_2$, 500 uM of dATP, dGTP, dTTP, and 50 uM dCTP. Twenty micro-Curies of $[32]$P dCTP was added to monitor the reaction. Cloned MLRV reverse transcriptase was added to the concentration of 200 U per ug of CTV RNA. The reaction was stopped with the addition of 5 ul of 2 M EDTA, and the unincorporated nucleotides were removed using spin column chromatography with Bio-Gel P60 from Bio-Rad (Richmond, CA 94804) as the column matrix gel. The cDNA:RNA hybrid molecules were ethanol precipitated and then tailed with dCTP using terminal deoxy-nucleotidyl transferase to catalyze the reaction. The reaction was stopped with the addition of 5 ul of 0.2 M EDTA, the unincorporated nucleotides were removed by spin column chromatography. The RNA-cDNA hybrids were ethanol precipitated, resuspended in water, and annealed to pUC-19 which had been restricted at the Pst I site and tailed with dGTP. The annealing conditions were as described by Cann *et al.* (7). The annealed plasmids were transformed into cells of *E. coli* which had been made competent by treatment with calcium chloride (34).

Analysis of the clones

Colony hybridization of the bacterial clones was done using procedures of Maniatis *et al.* (34). The plasmids were purified using an alkaline extraction procedure described by Promega (41). The inserts were cut with restriction enzymes and sized on 1% agarose gels. Conditions of Maniatis *et al.* (34) were followed for Southern blotting. The inserts were purified on agarose gels by the method described by Dretzen *et al.* (17). These purified fragments were nick-translated with $[32]$P dCTP as a label and used in Southern and Northern hybridization analyses using protocols described of Maniatis *et al.* (34). The filters were hybridized overnight in a solution containing sodium chloride

and sodium citrate (6X SSC), 5X Denhardt's solution, and salmon sperm DNA. The filters were rinsed for 5 minutes at room temperature and then for 15 minutes at either 50 C or 55 C in 2X SSC containing 0.5% SDS. The final rinse was for 15 minutes at either 50 C or 55 C in 0.5X SSC containing 0.5% SDS.

Subcloning and sequencing

The regions to be sequenced were subcloned by cutting the DNA with restriction enzymes, and the DNA fragments were recovered from agarose gels using NA 45 cellulose membranes (17). The cDNA inserts were ligated into the pGEM™ vectors. When ligating inserts into a pGEM™ vector at a single restriction site, the vector was dephosphorylated by the procedure of Maniatis *et al.* (34) for recessed ends using calf alkaline phosphatase. The protocol for denaturing the ds-DNA plasmids for sequencing was described by Promega Biotec (41). The dideoxy-chain terminating method of sequencing (46) was followed, and the reaction products were separated on a 6% polyacrylamide gel containing 7 M urea.

*In vitro* transcription and translation

The pGEM™ vectors with CTV-cDNA inserts were also used to generate single-stranded RNA. The RNA transcripts were initiated from either the Sp-6 or T-7 promoter and for some experiments were labelled with $^{[32]}$P-ATP, and used as hybridization probes. *In vitro* translation experiments using a rabbit reticulocyte lysate (16) were also attempted.

## Results

Complementary DNA cloning of CTV

Complementary DNA libraries were prepared to the T-30 and T-36 strains of CTV, and the library prepared from the T-30 RNA contained approximately 230 clones. About 40 of these clones have been sized on agarose gels and the average length of the cDNA inserts was 300-400 bases. Nearly 200 of the clones

hybridized to the cDNA insert of the clone designated 30F6. The cDNA insert was purified from an agarose gel and labeled with $[^{32}]$P dCTP by nick translation. In Northern blot analysis at 55 C, the 30F6 probe hybridized with T-30 RNA but only weakly with T-36 RNA (Fig. 6). This clone was approximately 650 bases in length, and the restriction map of the insert is shown in Figure 7. Portions of the clone 30F6 were sequenced and the nucleotide sequence and the predicted protein encoded by this region of the CTV genome are shown in Figure 8.

The library prepared from the CTV strain T-36 RNA contained over 500 clones. Selected clones were sized on agarose gels after they had been digested with the restriction enzyme Pst I, and the average length was 800 to 1000 bases. The two clones, designated P6A6 and P5D8, represented approximately 70% of the CTV genome. The clone P6A6 contained a cDNA insert of approximately 7350 bases, and the clone P5D8 contained an insert of approximately 8500 bases (Fig. 9). The extent of the homology of these clones was determined by both restriction site mapping and Southern hybridization analyses. They were homologous for 2200 bases and had five common restriction sites (Fig. 9). There was one restriction site polymorphism (the Sma I site) mapped at base 8300 on clone P5D8 (Fig. 9).

After restricting the clone P6A6 with Pst I, there were two cDNA fragments of approximately 2350 and 5000 bases as well as the vector band of 2750 bases (Fig. 9). The cDNA fragments were purified from agarose gels, radiolabelled by nick translation, and hybridized with the library of clones. Nearly 400 of the clones hybridized with the 2350 base fragment and about 100 with the 5000 base fragment. A 4600 base cDNA fragment from the P5D8 clone which had been cut with Xba I and Pst I was purified and radiolabelled. The

only clones besides the homologous P5D8 in T-36 cDNA library which hybridized with this fragment were P2C10, P2H5, and P5G9, and these clones were mapped to internal regions of P5D8. This was the evidence for mapping the P6A6 clone at the 3' proximal region of the CTV-RNA.

The 2000 base CTV-cDNA insert from the clone P2D10 and a 5000 base insert from the clone P6A6 were ligated into pGEM™. The RNA transcripts generated from the P2D10 construction contained a predominate species estimated to be 2100 bases (full length) on a glyoxal agarose gel. The P6A6 construct did not produce a predominate species and most of the transcripts appeared to terminate prematurely. The RNA transcripts from both strands of the P6A6 construct were hybridized with CTV-RNA at 50 C, and this experiment confirmed the 5' and 3' orientation of the clone in relation to the CTV-RNA (data not shown). Attempts to translate the RNA transcripts *in vitro* failed.

The region of the clone P6A6, which putatively represents 3' proximal terminus, was subcloned for sequencing. Figure 10 shows an autoradiogram of two P6A6 subclones, and the nucleotide sequence and the expected protein encoded by the subclone A6Sma are shown in Figure 11. Additionally the 3' junction of the clone P6A6 and vector was sequenced. The sequence at the junction could not be read accurately but it did contain a poly (G) region that appeared to be followed by a poly (T) region (data not shown). An oligonucleotide consisting of 5'-GGGGGGGTTT-3' was obtained and used as a primer. A sequence reaction was primed but it still contained many shadow bands which made the sequence ambiguous.

The amino acid composition of the predicted proteins from subclones of 30F6 and P6A6 was compared with the amino acid composition of the coat protein (table 1). While there were some similarities between the amino acid


Figure 6. An agarose gel and a Northern blot analysis of CTV-RNA. The CTV-RNA of strains T-30 (lanes 30) and T-36 (lanes 36) was denatured with glyoxal, subjected to electrophoresis on agarose gels and strained with ethidium bromide. The ethidium bromide stained bands are white on a dark background The lane m contains single-stranded RNA standards of 9.5, 7.5, 4.4, 2.4, and 1.4 kilobases from top to bottom. respectively. The Northern blot of the T-30 and T-36 RNA that has been hybridized with $^{[32]}$P-labeled clone 30F6 (from the T-30 cDNA library) is the section with the light background. Although it is not apparent in the photograph the clone 30F6 hybridized weakly with the T-36 RNA.

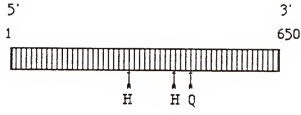


Figure 7. Restriction site map of the CTV T-30 cDNA clone 30F6. The position of the restriction sites mapped to the clones are designated using the 5' end as base 1 and the 3' end as base 650. The restriction site Sal I (Q) is at base 460, the restriction sites Hae III are at bases 320 and 444. This clone is inserted at the Pst I site in the vector pUC-19 and the Hind III site in the polylinker region is at the 5' terminus.

GAA CUA AAC GUG CCU GUU GUG AAC ACA ATA CAU GAA GUU CAA GGG GAA ACG
GLU LEU ASN VAL PRO VAL VAL ASN THR ILE HIS GLU VAL GLN GLY GLU THR

UAU AAG AAG GUC CGU UUG GUA AGG UGC AAA UAC CAA GAG GAC ACU CCU UUC
TYR LYS LYS VAL ARG LEU VAL ARG CYS LYS TYR GLN GLU ASP THR PRO PHE

UGU UCG GAU AAU CAU GUC GUU GUG GCG UUA ACC AGG CAU GUC GAC UCC UUA
CYS SER ASP ASN HIS VAL VAL VAL ALA LEU THR ARG HIS VAL ASP SER LEU

ACU UAC UCU GUU UUG AAU.AGU AGG AGA UAC GAC AAG ACU GCU UCU AAU AUA
THR TYR SER VAL LEU ASN SER ARG ARG TYR ASP LYS THR ALA SER ASN ILE

GAU GAA GCG AGG GAG AUU UUU GAC AAA UUU CGU UCG ACG AAC CAU UCU CAC
ASP GLU ALA ARG GLU ILE PHE ASP LYS PHE ARG SER THR ASN HIS SER HIS

GGU UCC UCA ACG UUA GAA TGC UAC CUC GAA AAG UAU CCU ACU GAA UAU
GLY SER SER THR LEU GLU TRP TYR LEU GLU LYS TYR PRO THR GLU TYR

AAG GGU AGC AAA GCA UCU UCG GCA CCG TTA
LYS GLY SER LYS ALA SER SER ALA PRO LEU

Figure 8. The RNA sequence and the predicted viral-encoded protein of the CTV T-30 cDNA clone 30F6. The sequence contains an open reading frame of 330 bases which predicts a protein of at least 111 amino acids.


Figure 9. A autoradiogram of sequencing reactions of portions of the two subclones from the T-36 cDNA clone P6A6. The A6Sma subclone reactions began at the P6A6 Sma I site which was at the position 12,800. The subclone O2A7 is a cDNA insert which was expressed in *E. coli*. The small letters represents the reactions of the four bases and contain the corresponding dideoxy-chain terminating base.

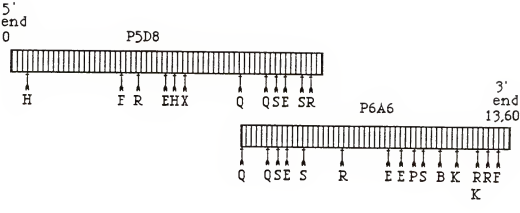


Figure 10. Restriction site map of the CTV T-36 cDNA clones P5D8 and P6A6. The position of the restriction sites mapped to the clones are designated using the 5' end as base 1 and the 3' end as 13,600. The Hind III sites (H) are at bases 400 and 4500, the Sph I sites (F) are at base 3100 and 7650, the Sma I sites (R) are at bases 3500, 8200, 9100, 11,300, and 12,800 and 13,100, the Eco RI sites (E) are at bases 4300, 7700, 10,400, and 10,750, the Xba I site (X) is at base 4600, the Sal I sites (Q) are at bases 6,300 and 7,000, the Sst I sites (S) are at bases 7250, 8000, and 11,400, the Pst I site (P) is at base 11,200, the Bam HI site (B) is at 11,850, and the Kpn I sites are at bases 12,300 and 12,750. The Sma I site at base 8,200 is unique to the P5D8 clone. All positions are approximate and there are additional restriction sites for Hind III, Sph I, Kpn I, and Eco RI whose positions have not been unambiguously mapped. These clones are in the vector pUC-19 and the Hind III site in the polylinker region is at the 5' termini of both clones. They are inserted at the Pst I site.

GG UAC CCA CCU AAA GAA AAA GGA AAC UCA CCA CUC ACU GGU AAA AGA GAA GAG
TYR PRO PRO LYS GLU LYS GLY ASN SER PRO LEU THR GLY LYS ARG GLU GLU

AGA AAG GAC CAA AAG AGG GCC CCG GGG CCG GAA UGC GGU AGG GUC UUC AAG CCC
ARG LYS ASP GLN LEU ARG ALA PRO GLY PRO GLU CYS GLY ARG VAL PHE LYS PRO

CGC GCU CGA UUC UCG AGA UUC AUG GGC CGU CUC GCG AAG AUC UUC GAU CCG AAC
ARG ALA ARG PHE SER ARG PHE MET GLY ARG LEU ALA LYS ILE PHE ASP PRO ASN

CUU CGC AUC UAC UCU CUC UUA CAC UUA AAG GUU GCU UUG CUU CGU UAU CGC CCC
LEU ARG ILE TYR SER LEU LEU HIS LEU LYS VAL ALA LEU LEU ARG TYR ARG PRO

UCG CUA CUG CUC AAC CUC GUA UCG CAU UUA UUC UUG CCG GCC CUU CCA GAU
SER LEU LEU LEU ASN LEU VAL SER HIS LEU PHE LEU PRO ALA LEU PRO ASP

UCC AAU UCC UUA UUG AGA UCG AGA UCU UGU UCC AUU AGA CCU AGU UUG
SER ASN SER LEU LEU ARG SER ARG SER CYS SER ILE ARG PRO SER LEU

GUC AGA UCA GUU CCA UAA UCUACGUUUGCCCCGGG
VAL ARG SER VAL PRO STOP

Figure 11. The RNA sequence and the predicted viral-encoded protein of the CTV T-36 cDNA subclone A6Sma, mapped from the Kpn I at base 12,750 to the Sma I restriction site at 13,100.

Table 1. The molar ratios of amino acids as predicted from sequence data of the CTV T-36 cDNA clone A6Sma and the CTV T30 clone 30F6 compared with the amino acid analysis of CTV capsid protein(a) (CP 1)

| Amino acid | Molar ratios | | |
|---|---|---|---|
| | A6Sma | 30F6 | CP1 |
| LYS | 7 | 8 | 17 |
| HIS | 2 | 5 | 4 |
| ARG | 15 | 7 | 8 |
| ASP | 2 | 6 | 22 |
| THR | 1 | 9 | 12 |
| SER | 12 | 12 | 16 |
| GLU | 4 | 9 | 13 |
| PRO | 10 | 4 | 5 |
| GLY | 5 | 3 | 24 |
| ALA | 5 | 5 | 16 |
| CYS | 2 | 2 | 1 |
| VAL | 5 | 11 | 13 |
| MET | 1 | 0 | 1 |
| ILE | 3 | 3 | 8 |
| LEU | 19 | 8 | 20 |
| TYR | 3 | 6 | 4 |
| PHE | 5 | 3 | 4 |
| TRP | 0 | 1 | NA |
| GLN | 1 | 2 | NA |
| ASN | 4 | 6 | NA |
| Total AA | 106 | 111 | 188 |

a: As reported by Lee *et al.* (32).

composition of the CTV coat protein and the predicted protein encoded by clone 30F6, there were also discrepancies The predicted protein encoded by the subclone A6Sma has several major differences in amino acid composition as compared with the CTV coat protein.

## Discussion

There is a significant difference in the length of the clones in the T-30 and T-36 libraries. One difference was to the source and age of the reverse transcriptase used in the preparation of these libraries. For the T-30 cloning, AMV reverse transcriptase which had been stored frozen at -80 C for two years was used and for the T-36 cloning a recently purchased lot of cloned MLRV reverse transcriptase was used. Because the majority of the cDNA inserts were short and homologous with the clone 30F6, the T-30 library did not appear to represent a large percentage of the CTV genome. The library prepared to T-36 RNA represented 70% of the CTV genome. While it is possible a larger percentage of the CTV genome was represented by clones in the library, only three clones shared homology with the 5' proximal region of the clone P5D8 and these mapped internally. At the 3' proximal region several clones were shown to start at approximately the same position as the clone P6A6. No clones were found to be 3' extensions of the clone P6A6, but there were many homologous clones that were not sized or mapped.

The orientation of the 5' and 3' direction of the clones was determined by three types of data. The cloning procedure used would be expected to be biased towards 3' end clones and the majority of the clones hybridized with the clone P6A6 but not with P5D8. Single-stranded RNA transcripts which were synthesized from the pGEM™ clone with a P6A6 cDNA insert were used as probes to detect CTV RNA in Northern blot analysis. Only the complementary

strand hybridized with the CTV RNA. Finally the putative 3' junction of the clone P6A6 and vector contained sequences that were difficult to read. These are typical of regions containing poly (G) and poly (T) sequences (40). Areas which contain poly (G) and poly (T) regions cannot be sequenced with either the enzyme Klenow or reserve transcriptase. Since the oligonucleotide (8G,3T) primer worked, it is probable that the reaction started at the junction of the vector and cDNA insert. The synthetic oligonucleotide needed to be purified on a PAGE-urea gel to be a more suitable primer.

The sequence data are incomplete in that only relatively short regions have been sequenced unambiguously. The resolved open reading frame in the subclone A6Sma consisted of at least 327 bases. If an open reading frame is more than 300 bases in length, there is less than a 1% chance that it is a fortuitous open reading frame (54). The beginning of the open reading frame was in an area which has not been sequenced, but the end of the open reading frame was determined. The orientation of the clone was known, and the predicted open reading frame was on the messenger-sense strand. The other possible reading frames had multiple stop codons and the sequence data appeared to be unambiguous. Still caution must be used in interpreting sequence data (28), especially the presence of stop codons when sequencing RNA viruses. Doughtery *et al.* (15) used sequence data to predict an alternative mode of potyvirus capsid expression based on the presence of stop codons. Data by the same author as well as data from other laboratories (2,3,16) indicate that potyviruses synthesize a single polypeptide, and post-translationally process the polypeptide into the protein products. To achieve a higher degree of confidence with RNA viruses, it is necessary to sequence either two independent clones or one clone and directly sequence the viral genome.

The clone 30F6 contained an open reading frame of 330 bases. The 3' end of this open reading frame was at the junction of the clone and vector. The remaining 320 bases of the clone have only been sequenced in a single direction. When the entire sequence of the clone is completed, a more accurate comparison of the predicted protein encoded by 30F6 and the capsid protein of CTV can be made.

# CHAPTER 4
# THE EXPRESSION OF CITRUS TRISTEZA VIRAL-ENCODED PROTEINS IN *ESCHERICHIA COLI*

## Introduction

The identification of viral-encoded proteins and the production of antisera to these citrus tristeza virus (CTV) putative proteins was attempted by expressing portions of the CTV-cDNA in *E.coli*. The expression of plant viral proteins in *E. coli* has been demonstrated when antisera to the protein is available (37). Many expression systems require that either antisera is available to detect the foreign protein or that the sequence of the gene to be expressed is known (54). Since no antisera to the non-structural proteins of CTV are available, the expression plasmids (pORF) developed by Weinstock *et al.* (55), which use ß-galactosidase as a selectable marker, were chosen for this study. These expression plasmids contain the ß-galactosidase gene out of reading frame. When a DNA insert contains an open reading frame that is of a length that puts the ß-galactosidase gene in frame, then a hybrid protein (fusion protein) of ß-galactosidase and the protein encoded by the open reading frame of the cDNA is produced. These ß-galactosidase fusion proteins are normally enzymatically active and blue colonies are produced on media containing 5-bromo-4-chloro-3-indolyl-ß-D-galactosidase (X-gal). Also this vector system contains two strains of *E. coli* , one strain produces the fusion protein at low levels and the other expresses the fusion protein at high levels. This increases the chance of success since many foreign proteins are toxic to *E. coli* (54).

A region of a CTV-cDNA clone, which represented about 30% of the CTV genome, was subcloned into pORF expression system. Fusion proteins, which were expressed in *E. coli* , were identified and analyzed to determine if portions of these proteins contained CTV specified polypeptides.

## Materials and Methods

### Materials

Open reading frame plasmids pORF-1 and pORF-2 and the *E. coli* strains MH-3000 and TK-1046 were from the American Type Culture Collection (Rockville, MD 20852). The ß-galactosidase antiserum was obtained from Cooper Biomedical, Inc. (Malvern, PA 19355). The restriction enzymes, Klenow, DNA polymerase I, T-4 ligase, 1 kilobase and 123 base ladders were from Bethesda Research Laboratories, Inc. (Gaithersburg, MD 20877). Dideoxynucleotides, pGEM™ vectors, Sp6 and T-7 oligonucleotide primers were purchased from Promega Biotec (Madison, WI 53711). Calf alkaline phosphatase and X-gal were obtained from Boehringer Mannheim (Indianapolis, IN 46250). Nitrocellulose membranes and NA-45 cellulose membranes were from Schleicher and Schuell (Keene, NH 03431). The gamma globulin free bovine serum albumen and goat anti-rabbit alkaline phosphatase were purchased from Sigma (St. Louis, MO 63178). The p-nitroblue tetrazolium chloride and p-toluidine salt of 5 bromo-4-chloro-3-indolyl phosphate were purchased from Bio-Rad (Richmond, CA 94804).

### Preparation of expressed cDNA sequences

A 5000 base region of a T-36 CTV-cDNA clone was purified from the pUC-19 vector sequences by recovery of CTV-cDNA from agarose gels using NA-45 membranes (17). This cDNA fragment was cut with either the restriction enzyme Hae III or Alu I. The preparations were then extracted with phenol:chloroform and ethanol precipitated. The pORF-1 or pORF-2 vectors

were restricted at the Sma I site and dephosphorylated with the enzyme calf alkaline dephosphatase (34). All four combinations of the pORF vectors and restriction digest fragments were ligated and transformed into cells of *E. coli* strain MH-3000 which had been made competent by treatment with calcium chloride (34). Blue colonies were selected from which plasmids were purified using an alkaline purification proccdure (41).

Detection of expressed fusion protein

To produce high levels of fusion proteins, the purified plasmids were transformed to the *E. coli* strain TK-1046. The cells were grown at 28 C until the culture at A=550 nm was 0.2 and then incubated at 37 C for 2 hours (54). The cells were collected by centrifugation and the resulting pellet was resuspended in Laemmli's dissociation solution and boiled for 5 minutes. The lysed cells were centrifuged for 15 minutes in a microfuge. The supernatants were loaded onto a 7.5% SDS-polyacrylamide gel with a 4% stacking gel using the procedure described by Laemmli (29). The gels were run for 15 hours at 50 volts. The proteins were either visualized by staining the gels with Comassie blue or transferred to nitrocellulose membranes for Western blot analysis (48). The transfer conditions were 5 hours at 80 volts at 5 C in buffer consisting of 25 mM Tris, 192 mM glycine, and 20% methanol using a TE transfer unit from Hoefer Scientific Inc. (San Francisco, CA 94107). The membranes were soaked in 0.9% NaCl and 20 mM Tris, pH 8.2 (TBS) containing 1% BSA. The membranes were then incubated in TBS containing 0.1% BSA (TBS-BSA) and a 1/500 dilution of an antiserum specific for ß-galactosidase for 1 hour. After three washes with TBS-BSA, the membranes were incubated for 1 hour in TBS-BSA containing 1/1000 dilution of goat anti-rabbit antisera conjugated with alkaline phosphatase. After three washes with TBS-BSA, the membranes were rinsed with 1 M diethanolamine, pH 9.6. The development

was done at room temperature in a solution consisting of 20 ml of 1 M diethanolamine, 2 mg of p-nitroblue tetrazolium chloride, 1 mg of p-toluidine salt of 5 bromo-4-chloro-3-indolyl phosphate, and 40 ul of 2 M magnesium chloride and was stopped by rinsing the membrane with water.

Purification of the fusion protein for antisera production

The fusion protein, produced by the clone designated O2A7, was eluted from SDS-PAGE gel and used as an immunogen to produce antibodies. The protein bands in the SDS-PAGE gel were visualized by soaking the gel in cold 0.3 M KCl. The area containing the fusion protein was sliced from the gel, and protein was eluted from the pulverized gel band with water. The eluted protein was analyzed for size by SDS-PAGE, transferred to nitrocellulose membranes and detected using ß-galactosidase antiserum to assure it was the fusion protein before using it as an immunogen. The rabbit was given three injections at two week intervals for production of the polyclonal antiserum.

Sizing and sequencing of the CTV-cDNA

The CTV-cDNA inserts ligated into the pORF vectors at the Sma I site were flanked by Bam HI sites. The plasmids which produced fusion proteins were cut with Bam HI restriction enzyme and sized on 2% agarose gels. These DNA bands were recovered from agarose gels using NA-45 cellulose membranes. The pGEM™ vectors, were cut at the Bam HI site and dephosphorylated using the procedure of Maniatis *et al.* (34) with calf alkaline phosphatase and ligated with the cDNA inserts. The protocol for denaturing ds-DNA plasmids was described by Promega Biotec (41). The dideoxy chain termination method was used for the sequence reactions (46). The reaction products were run on 6% polyacrylamide gels containing 7.0 M urea.

## Results

The CTV-36 clone, designated P6A6, consisted of approximately 7350 bases of CTV-cDNA and 2700 bases of the vector pUC-19. This clone was cut with the restriction enzyme Pst I at three sites. A 2700 base vector band and two bands of 5000 and 2350 bases consisting of the CTV-cDNA were apparent after agarose gel analysis (data not shown). The 5000 base fragment of the clone was cut into many small bands with either Alu I or Hae III. These fragments were ligated into the pORF 1 and 2 vectors at the Sma I site and used to transform *E. coli* strain MH-3000. Most of the resulting colonies were white, but over 30 blue colonies were selected for further analysis. At least five different inserts were identified using the insert size and the vector as criteria (Fig. 12). These plasmids were transferred to *E. coli* strain TK-1046 and evaluated for the presence of fusion proteins. Several of the clones produced a protein of approximately Mr 125-130 X $10^3$ which was not present in the control *E. coli* strain TK-1046 containing pORF-1 plasmid without an insert (Fig. 13). All but one of these proteins were identified as fusion proteins, because they reacted with ß-galactosidase antiserum and were of higher molecular weight than native ß-galactosidase (Fig. 13). The clone, designated O1H3 and approximately 330 bases in length, produced light blue colonies on media containing X-gal in both the MH-3000 and TK-1046 strains of *E. coli*. A protein of approximately Mr 130,000 was detected on SDS-PAGE gels which were stained with Comassie blue, but this protein did not react with ß-galactosidase antiserum.

Two clones, designated O2A2 and O2A7, were sequenced and they were identical cDNA inserts of 100 bases. The complete sequence and the predicted amino acids expressed as part of the fusion protein are shown in Figure 14.

The fusion protein expressed by bacterial cells containing the clone O2A7 was eluted from SDS-PAGE gels and used to produce a polyclonal antiserum in a

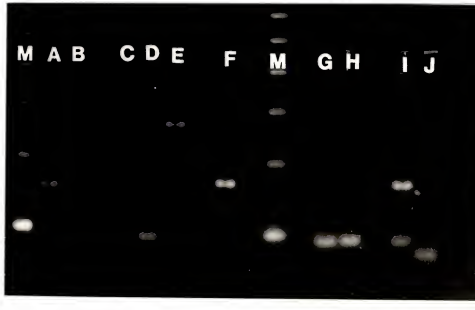


Figure 13. An agarose gel size analysis of CTV-cDNA inserts in pORF plasmids that were selected as expressing fusion proteins. The plasmids were cut with Bam HI, run on 2% agarose gels, and stained with ethidium bromide. Lane A contains the CTV-cDNA insert from the plasmid designated O1A1, lane B O1A3, lane C O1A14, lane D O1A28, lane E O1H3, lane E O2A4 and O2A21, lane G O2A9, lane H O2A11, lane I O2A4 and O2A21, and lane J O2A26. The lanes M contain markers of 123, 246, 369, and 492 bases as read from the bottom of the gel.

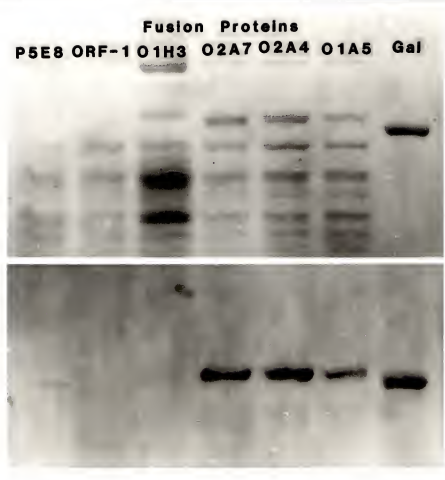


Figure 14. An SDS-PAGE gel and Western blot of fusion proteins expressed by *E. coli* cells containing pORF plasmids with CTV-cDNA inserts. The top picture is a SDS-PAGE gel after staining with Comassie blue. The bottom picture is a Western blot analysis of the top gel that was reacted with antiserum to ß-galactosidase and followed with goat-anti-rabbit antiserum conjugated with alkaline phosphatase. The ORF-1 lane is the pORF-1 without a cDNA insert, O1H3 is the pORF-1 with a 320 base cDNA insert, O2A7 and O2A4 are pORF-2 with a 100 base cDNA insert, and 01A5 is pORF-1 with a 100 base insert. The lane P5E8 was a pUC-19 clone with a 1600 base cDNA insert that produced light blue colonies.

CCC UGC CAA AAG AGA UUU UCC UCC GUU UGU UUU UUG GGG CUA GAA GAU
PRO CYS GLN LYS ARG PHE SER SER VAL CYS PHE LEU GLY LEU GLU ASP

AAG AUU UAU GCA GUC UAU AGA AAA AAA AAA AAA GAA AAA AAG
LYS ILE TYR ALA VAL TYR ARG LYS LYS LYS LYS GLU LYS LYS

AAG UAU UAU GAA
LYS TYR TYR GLU

Figure 15. The sequence and predicted amino acid of the cDNA insert of the clone O2A7. The sequence was a subclone of P6A6 which ligated into pORF-2 and the clone was selected as expressing a fusion protein. The insert was then transferred into the pGEM-3 plasmid for sequencing.

rabbit. This antiserum reacted with several *E. coli* proteins as well as ß-galactosidase. This antiserum has not been determined whether or not it reacted with a CTV encoded protein, or if it reacted with the protein predicted from the sequence of the O2A2 insert.

## Discussion

Most of the clones which expressed fusion proteins contained short CTV-cDNA inserts and this increased the probability that fusion proteins were expressing fortuitous open reading frames and not viral proteins encoded by the CTV genome. If the expressed open reading frame is over 300 bases in length, the probability is less than 1% that the reading frame is fortuitous (54). The sequencing of the expressed inserts of O2A2 and O2A7 did not add confidence that they were representative of an expressed region of the CTV genome. The long sequence of poly (A) (Fig. 15) is similar to the poly (A) tract that is found in the non-coding region between the two genes encoded by the RNA 3 of brome mosaic virus (1). There was no evidence that the antiserum made from the fusion protein expressing this sequence was specific for a CTV protein. The longest insert of the clone generated was 330 bases in the clone O1H3. It appeared to express a protein of the proper size for a fusion protein, but since the protein did not react with a ß-galactosidase antiserum, it was not sequenced or used as an immunogen.

Use of the *E. coli* expression vectors was difficult and the results were confusing. Maintenance of stable clones was difficult especially when the clones were in the high expression strain TK-1046. White colonies would often appear in the middle of blue colonies when clone O2A2 or O2A7 were cultured at 37 C. The most probable explanation for this was the loss of the pORF in these white colonies.

To make rapid progress in preparing antisera to CTV proteins, a more systematic approach for the production of fusion proteins is needed. There is the need to sequence more of the CTV genome and to identify additional restriction sites and the extent of the open reading frames. Then specific constructs can be made which should produce fusion proteins with the pORF vector system. The pORF vectors can be improved by the addition of a polylinker sequence such as the sequence which has been engineered into the pUC-19 plasmid. This would give greater flexibility for making constructs which express fusion proteins. For the areas of the genome which have been sequenced, another approach involving the synthesis of synthetic polypeptides based on the predicted sequence encoded by the viral genome could be used (39). These polypeptides could be attached to a carrier protein and used as immunogens to make antibodies.

# CHAPTER 5
# CONCLUSIONS

The successful production of cDNA clones to portions of both the CTV and CVV genome was accomplished using a cDNA:RNA hybrid method of cloning described by Cann *et al.* (7). Use of this procedure necessitated the addition of poly (A) tails to the viral RNA. Despite this additional step, this cloning procedure involves a minimum number of manipulations. The polyadenine tailing procedure adds poly (A) tails to free 3' hydroxyl termini of RNA (14), and using this procedure, clones to internal regions of viral RNA can be generated from fragmented RNA (7,47). The CTV RNA shears easily and internal cDNA clones were produced. Approximately 70% of the CTV genome was represented by the cDNA clones in the T-36 library. Several modifications of the cloning procedure were tried and the elimination of several phenol:chloroform extraction steps increased the number of cDNA clones. The change of salt from NaCl to KCl was needed when sodium pyrophosphate was added to reaction to prevent the precipitation of the RNA. Sodium pyrophosphate was added to the reaction because it has been reported to prevent the RNAse H type activity of reverse transcriptase and consequently to increase the average length of cDNA clones (36).

The cDNA clones were used as probes to detect both homologous as well as heterologous strains of the viruses. The CVV-cDNA clones were useful to characterize the CVV RNA 4 and RNA 4a species and to demonstrate that these species are homologous with the CVV RNA 3 and RNA 2, respectively. Also the

CVV-cDNA clones were highly specific in that no hybridization could be detected with the heterologous strain CVV-FM or with the serologically related virus CLRV. The CTV-cDNA clones were also strain specific and they hybridized only with some of the other CTV strains tested (data unpublished). The specificity was similar to the selectivity that has been demonstrated by Rosner *et al.* (42,44). These probes could be useful in cross protection experiments to determine if the severe challenge strains become systemically established.

The attempts to detect expressed viral-encoded CTV proteins in *E. coli* were complicated because the only antisera available was to the CTV coat protein. The pORF vectors (54) have a selectable marker which indicates that a fusion protein is being expressed and that the cDNA insert has an open reading frame. One problem with this vector system is that there is an 11% probability that a randomly generated insert containing an open reading frame will be expressed as a selectable trihybrid fusion protein. In the expression experiments, four base specific restriction enzymes were used and these tend to generate short DNA fragments (probability of one restriction site every 256 bases). The longest cDNA insert selected, using this expression system, was 320 bases and this clone was not confirmed to express a trihybrid fusion protein. The fusion protein produced by one of the 100 base clones was used to prepare an antiserum, however, this antiserum has not been shown to be specific for a viral-encoded protein.

Since the protein expression system yielded ambiguous results, it was desirable to sequence portions of the cDNA clones, because through nucleotide sequencing, open reading frames as well as the positions of restriction sites can be identified. The nucleotide sequence of a viral genome can also be used

to predict the probable transcription and translation strategy of a virus (27). After sequencing about 350 bases of two CTV cDNA clones, two open reading frames and several restriction sites were identified. There still is not enough sequence data to predict the insertion of one of the cDNA clones into the pORF vectors to express a fusion protein. Additional sequencing would increase the probability of making such a prediction.

*In vitro* expression of RNA generated from cDNA clones has been used to study the genomic organization of plant viruses (51). Two T-36 cDNA clones were transferred into pGEM™ to get the clones to express *in vitro* (16). Single-stranded-RNA transcripts were generated from both strands of the cDNA clones using either the Sp-6 or T-7 bacteriophage promoter (35). These vectors efficiently produced RNA transcripts, which were useful in determining the 5' and 3' orientation of the cDNA clones, but the RNA transcripts did not translate *in vitro*. The pGEM-3 is not an appropriate vector for the initiation of translation from the 5' end of the RNA transcript generated from the T-7 promoter because the fifth codon is a TAG stop codon (41). There is only a 33% probability of getting suitable transcripts when randomly spliced cDNA fragments containing an open reading frame are inserted into the Sp-6 promoter. The addition of a ribosome binding site and a start codon into either the cDNA clones or the vector system should make the transcription and translation of viral cDNA clones easier. The knowledge of the nucleotide sequence of the viral RNA would simplify the construction of clones which could be translated *in vitro*.

The pGEM™ vectors proved to be efficient for ds-DNA sequencing, because the clones in these vectors could be sequenced from both directions. Although only limited portions of the clones 30F6 and P6A6 have been sequenced,

already the primary structures of two polypeptides have been predicted. Since both predicted open reading frames were more than 300 bases in length, there was less than a 1% chance that they were fortuitous open reading frames (54). The protein encoded from the P6A6 clone was shown to be encoded by the strand representing the viral RNA. The open reading frame of 327 bases ended with a TAA stop codon. There were multiple stop codons in the other reading frames. Further sequencing needs to be done to determine if there is a start codon downstream from the stop codon. This area is in the 3' proximal area of the T-36 clone map. The 3' terminus is the region of the genome encoding the coat protein of many plant viruses (13,38). The sequencing of the clone 30F6 should be completed, since the predicted protein from this clone has some similarities with the amino acid composition of the CTV coat protein. Only one additional subcloning is needed to finish sequencing the entire clone.

The cDNA libraries to CVV and CTV should be valuable tools for continuing the studies on the molecular biology of these viruses. Much has been learned about the expression of viral-encoded proteins in *E. coli* and the *in vitro* transcription and translation of viral RNA. Future experiments should be conducted using the nucleotide sequence data as the basis for construction clones which express CTV viral proteins. These experiments should yield fusion proteins that unambiguously contain CTV-encoded proteins, which could then be used as immunogens. The identification of CTV non-structural proteins and the mapping of these proteins on the viral genome will help in the understanding of the molecular biology of the inadequately characterized closterovirus group.

APPENDIX A
PROCEDURES

Deoxynuclotide 3' tailing reaction

Add
1. 1 ug of vector or cDNA:RNA hybrid molecules in 38 ul of sterile water

2. 10 ul of 5 X tailing buffer

3. 1 ul of 100 uM dNTP

4. 5 units of deoxynucleotidyl terminal transferase (dTt)

Then incubate at 30 C for 30 minutes

Stop reaction with 5 ul of 0.2 M EDTA and then treat with Phenol:Chloroform. Then add 0.5 volumes of 7.5 M AmAcetate and 2.5 volumes of EtOH to precipitate the NA. The 5 X transcription buffer is supplied with the enzyme dTt from BRL (Gaithersburg MD).

Polyadenine Polymerase reaction adapted from Devos *et al.* (14)

Add
1. 2-5 ug of viral RNA in in 30 ul of water

2. 4 ul 1.0 M Tris-HCl pH 7.9

3. 1 ul 0.8 M $MgCl_2$

4. 2 ul 0.2 M $MnCl_2$

5. 10 ul NaCl 2.0 M

6. 0.5 ul Bovine serum albumin

7. 1 ul 100 uM ATP

8. 2 Units of poly(A) polymerase

Incubate at 30 C for 30 minutes

Then stop the reaction with 5 ul 0.2 M EDTA and treat with Phenol:Chloroform. Then add 0.5 volumes of 7.5 M AmAcetate and 2.5 volumes of EtOH to precipitate the RNA.

cDNA : RNA hybrid cloning adapted from Cann et al. (7).

cDNA synthesis

Add

1. 2 ug of polyA tailed RNA in 55 ul of water
2. 5 ul of 1.0 M Tris-HCl pH 8.3
3. 20 ul of 0.3 M $KCl_2$
4. 1 ul of 0.8 M $MgCl_2$
5. 1 ul of 0.2 M 2-mercaptoethanol
6. 1 ul of 0.2 M NaPyrophosphate
7. 0.5 ul of oligo dT 12-18 1 ug/ul
8. 5 ul of 10 mM dATP
9. 5 ul of 10 mM dTTP
10. 5 ul of 10 mM dGTP
11. 1 ul of 100 uM dCTP
12. 20 uCi [$^{32}$P] dCTP
13. 200 units / ug RNA of cloned reverse transcriptase from BRL.

Incubate for 15 minutes at 37 C.

14. 2.0 ul of 10 mM dCTP

Incubate for 45 minutes at 37 C.

Then stop the reaction with 5 ul of 0.2 M EDTA and run on Bio-rad P-60 Agarose column to remove unicorporated nucleotides and to remove enzyme activity. Add 0.5 volumes of 7.5 M AmAcetate and 2.5 volumes of EtOH to precipitate RNA:cDNA hybrids. Then follow the deoxynuclotide 3' tailing reaction. After completing the tailing reaction go to the annealing reaction.

Annealling reaction (7)

Add

1. 232 ul of water to resuspend the RNA:cDNA hybrids

2. 12.5 ul of 2 M NaCl

3. 5 ul of 0.5 M Tris, pH 8.0 and 10 mM EDTA

4. Heat to 65 C for 5 minutes and the put hybrids in a 45 C water bath for two hours. Then slow cool to room temperature and finally place on ice. Then follow the transformation procedure.

Preparation of competent cells (34)

1. Inoculate 250 ml of LB broth with 3 ml of an overnight cluture. Incubate and shake culture at 37 C until the culture reaches an optical density of 0.4 at 550 nm

2. Centrifuge the cell suspension at 3000 g for 5 minutes. Pour off the supernatant and resuspend the bacterial cells with 125 ml of 50 mM $CaCl_2$, 10 mM Tris, pH 8.0 that is at 0 C.

3. Centrifuge the cell suspension at 3000 g for 5 minutes. Pour off the supernatant and resupend the bacterial cells with15 ml of 15% glycerol, 50 mM $CaCl_2$, 10 mM Tris, pH 8.0 that is at 0 C. Pipet 1 ml aliquoits of the baterial cells suspension and freeze at -80 C.

Transformation procedure (34)

1. Thaw the competent cells in an ice slurry, and make aliquot 0.2 ml of the bacteral cells.

2. Add up to 50 ul of plasmid DNA and incubate at 0 C for 30 minutes.

3. Heat shock the bacterial cells at 42 C for 2 minutes.

4. Mix 2 ml of LB media containing 1% agar and pour onto LB petri plate containing when appropriate antibiotics and X-gal.

5. Incubate plates at 37 C.

LB media 10 g tryptone
5 g yeast extract
10 NaCl
1 liter of water

Alkaline lysis to purify plasmid DNA

1. Grow an overnight culture in LB media with the appropriate antibiotic.

2. Centrifuge 9 ml of the culture and centrifuge 5 minutes at 3,000 g.

3. Resuspend pellet in 600 ul of ice cold 50 mM glucose, 10 mM EDTA, 25 mM Tris, pH 8.0, and 4 mg/ml lysozyme

4. Let reaction proceed for 30 minutes on ice.

5. Add 1200 ul of freshly prepared 0.2 NaOH and 1% SDS. Invert tube to mix and incubate for 5 minutes.

6. Add 900 ul of ice cold potassium acetate, invert to mix, incubate for 30 minutes on ice. To 60 ml of 5 M potassium acetate, add 11.5 ml of acetic acid, and 28.5 ml water.

7. Centrifuge for 15 minutes at 10,000 g and transfer the supernatant to a fresh tube and repeat the centifugation.

8. Add 10 ul of a solution containing 1 mg RNase A and 10,000 units of $T_1$ and incubate at 37 C for 15 minutes.

9. Phenol:Chloroform extract twice.

10. Add 2 volumes of EtOH and precipitate DNA.

11. Resuspend pellet in 160 ul of $H_2O$, 40 ul of 4 M NaCl, and then add 200 ul of 13% PEG and incubate on ice for one hour.

12. Centrifuge at 12,000 rpm for 15 minutes, wash with 70% EtOH and dry the pellet. Resuspend in 50 ul of $H_2O$.

APPENDIX B
LIST OF CLONES

The library of CVV and CTV clones are stored at -20 in LB broth with 15%glycerol and in freeze broth at -80 in sterile microtiter plates. The beginning of a designation for the CTV clones (P1, P2, P3, etc.) refers to the plate number and the second designation (A6-H12) refers to the position in the microtiter plate.

Purified plasmids

T-36 cDNA clones in pUC-19

| | | |
|---|---|---|
| P1B3 | P3D5 | P4B11 |
| P3A7 | P6E8 | P4D11 |
| P2D10 | P6C9 | P6E4 |
| P1D12 | P6C5 | P6F12 |
| P4B7 | P5B1 | P6A6 |
| P5D8 | P3F7 | |

Clones which have been inserted into the pGEM™ vectors.

| | | |
|---|---|---|
| pGEM3 | pGEM4 | PG3O2A2 |
| PG3O2A7 | PG4D10 | PG3A6 large Pst fragment |

The clones in the pORF vectors that produce blue colonies when grown on media containing X-gal. The 01 or 02 designation indicates the pORF-1 or pORF-2 vector.

| Clone | Size | Clone | Size | Clone | Size |
|---|---|---|---|---|---|
| O2A1 | | 02A9 | 100 | 01A1 | 180 |
| 01H3 | 320 | 02A4 | 101 | O1A14 | 100 |
| O2A21 | 200 | O2A11 | 100 | O2A13 | |
| O2A6 | | 02A26 | 80 | O2H2 | |
| O1A3 | | 02A7 | 101 | 01A28 | 100 |
| O2A29 | | | | | |

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## BIOGRAPHICAL SKETCH

Lee Alexander Calvert was born in St. Petersburg, Florida, on August 13, 1951, and was raised in Louisville, Kentucky. He graduated from Louisville Country Day School in 1969 and completed his undergraduate degree in botany from Ohio Wesleyan University in 1973. After working for one year with the Environmental Protection Agency in Cincinnati, Ohio, he joined the Peace Corps as a health volunteer and worked for three years in the Republic of Korea. In 1978, he was employed in the Department of Plant Pathology at the University of Kentucky as a laboratory technician in Dr. S.A. Ghabrial's plant virology laboratory. In 1980, he started a degree program and obtained a Master of Science degree under the direction of Dr. Ghabrial in 1981. He was then employed as a biological scientist II by Dr. R.F. Lee at the University of Florida Citrus Research and Education Center in Lake Alfred, Florida. In the fall of 1983, he began his Ph.D. research under the direction of Dr. E. Hiebert and Dr. R.F. Lee at the University of Florida in Gainesville, Florida. He has accepted a postdoctoral research associate position with Dr. S. Lommel in the Plant Pathology Department at Kansas State University in Manhattan, Kansas.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Ernest Hiebert

Ernest Hiebert, Chairman
Professor of Plant Pathology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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